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The effect of Mongolian Medicine Chagan Gaoyou-4 Powder on bone mineral density induced by retinoic acid in rats

Tubuxin, Seesregdorj Suregjid, Dorjbat Sosorburam*

International School of Mongolian Medicine, Mongolian National University Medical Sciences, Ulaanbaatar 14210, Mongolia

* **Corresponding author:** Dorjbat Sosorburam, dorjbat@mums.edu.mn

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Abstract: Objective: To investigate the effects of Chagan-Gaoyou-4 powder on spine bone mineral density and serum estrogen and estrogen receptor alpha (ER α) and estrogen receptor beta (ER β) protein expression in bone tissue induced by retinoic acid in rats. **Methods:** Sixty 3-month-old SPF female SD rats were randomly divided into model group (retinoic acid group), normal group (SHAM group), Mongolian medicine group (Chagan-Gaoyou 4 group), and control group (Gushukang granule group). After 14 days of administration, spinal Bone Mineral Density (BMD) was measured, and external manifestations were observed. **Results:** Compared with the SHAM group, the BMD values of all medication groups decreased ($P < 0.05$, $P < 0.001$). Compared with the model group, the spinal BMD value of the Chagan Gaoyou-4 group was increased after 21 days of treatment, and the difference was statistically significant ($P < 0.01$, $P < 0.05$). After 21 days, the spinal BMD value of all treatment groups was increased ($P < 0.05$), the spinal BMD value was also increased, especially Chagan Gaoyou-4 group was more obviously increased ($P < 0.05$). Compared with the normal group, ER α and ER β levels in the model group were significantly decreased ($P < 0.001$), and ER α and ER β levels in all drug groups were significantly increased ($P < 0.001$) compared with the model group. After 21 days of treatment, there was no significant difference among the three treatment groups ($P > 0.05$). ER α and ER β levels in the Chagan Gaoyou-4 group were higher than those in the model group ($P < 0.05$), but there was no significant difference between the group and the Gushukang granule group ($P > 0.05$). The E2 content of the Chagan Gaoyou-4 group was higher than that of the model group ($P < 0.05$). The mRNA expression of estrogen receptor ER α and ER β in bone tissue of the left proximal femur was detected by RT-PCR. Compared with the model group, the expression of ER α and ER β mRNA in the Chagan Gaoyou-4 group and Gushukang granule group was increased. Compared with the model group, ER α mRNA expression was increased in the Chagan Gaoyou-4 powder medium-dose group. Compared with the Gushukang granule group, there was no significant difference in ER α mRNA expression in the Chagan Gaoyou-4 dose groups. Chagan Gaoyou-4 can up-regulate the expression of ER α and ER β induced by retinoic acid in rats, indicating that the Chagan Gaoyou-4 powder group may promote bone formation, regulate bone resorption, improve bone mineral density, and achieve the purpose of preventing and treating OP by increasing estrogen level, stimulating estrogen receptor, and increasing the expression of ER α and ER β in bone tissue. **Conclusion:** Chagan-Gaoyou-4 powder may have estrogen-like effects on the bone tissue of rats induced by retinoic acid and may increase the level of serum estrogen, ER α , and ER β protein expression, thereby improving the spinal BMD of experimental rats. The Chagan-Gaoyou-4 powder group could improve the general condition of osteoporosis induced by retinoic acid in rats.

Keywords: Chagan Gaoyou-4 powder; estrogen receptor ER α ; ER β ; estradiol; bone density

1. Introduction

Osteoporosis (OP) is one of the common chronic diseases of the elderly, which can lead to a decrease in bone density and bone quality, the destruction of bone microstructure, the increase of bone fragility, and the metabolic bone disease that is easy to fracture. According to the current survey, the total incidence of OP in people over 65 years old in China is 32.0%, 10.75% in males and 51.6% in females, with an increasing trend year by year [1,2], and OP leads to disability and even death. In recent years, more and more attention has been paid to the prevention and treatment of OP by traditional medicine. It is of great significance to find an alternative medicine in traditional Mongolian medicine for the prevention and treatment of osteoporosis.

Mongolian medicine Chagan Gaoyou-4 powder was published in the Chinese Medical Encyclopedia (Mongolian Medicine Volume), which is composed of Hanshuishi, Cistanche, rock sugar, and Digeda. Among them, Hanshuishi is rich in calcium sulfate [3–7], and cistanche extract has been proven to have estrogen-like effects [8–10], which can effectively prevent and treat OP [11–14].

Estrogen replacement therapy widely used in clinics, but more and more clinical and experimental studies have shown that the long-term use of synthetic estrogen drugs has many toxic side effects, increasing the incidence of breast cancer, endometrial cancer, cardiovascular disease, etc., and its application has been subject to many controversies [12]. Therefore, estrogen replacement therapy is no longer used as a first-line prevention and treatment method [11], but more suitable estrogen-like drugs can be found for the prevention and treatment of OP based on the mechanism of estrogen action. Tathagata originates from phytoestrogens and selective estrogen receptor modulators (SERMs) in Mongolian medicine. Although these drugs effectively inhibit osteoclast bone resorption, reduce bone loss, reduce the incidence of vertebral fracture in postmenopausal women, and increase BMD, their long-term use will increase the risk of venous blood embolism. Therefore, guided by the basic theory of traditional medicine, it is of great practical significance to explore more economical compounds as an alternative medicine for OP prevention and treatment from the treasure house of traditional Chinese medicine in China. This drug has a long clinical history serving Mongolian medicine, with not only good clinical efficacy but also advantages such as low price and few toxic side effects. I have conducted a clinical study on 60 cases of osteoporosis treatment in 2019 and found that Chagan Gaoyou-4 powder has no toxic side effects on the heart, liver, and kidneys of patients [15], except for myself, Wurihan [16] used Zhuang Xi-4 (Another name of Chagan Gaoyou-4) powder combined with calcium carbonate D₃ tablets to treat postmenopausal osteoporosis. It was found that Zhuangxi-4 powder combined with calcium carbonate D₃ tablets had a better effect on increasing bone density, bone metabolism indicators, estradiol, and improving clinical symptoms in postmenopausal osteoporosis than using calcium carbonate D₃ tablets alone, and all safety indicators showed no abnormalities.

In this study, a rat model of OP induced by retinoic acid was established, and the randomized controlled grouping principle was applied to conduct a comparison analysis with positive drugs, respectively. Spine bone mineral density, serum estrogen level, ER α , and ER β mRNA expression in bone tissue were detected. Chagan Gaoyou-

4 powder can increase the serum estrogen level, the mRNA expression level of ER α and ER β in femur bone tissue, and the bone mineral density of the spine in experimental rats. Therefore, the purpose of this study was to investigate the effects of Chagan Gaoyou-4 powder on BMD and estrogen levels, ER α , and ER β protein expression, and to provide evidence for the prevention and treatment of OP.

2. Materials and methods

2.1. Drugs and reagents

Chagan Gaoyou-4 powder (Preparation Room, Affiliated Hospital of Inner Mongolia Minzu University, batch No. M15060996); Tretinoin (Shaanxi Senfu Biotechnology Co., LTD., lot No. SCI120308-03); Estrogen (Nanjing Institute of Biological Engineering, Batch No. 20181222); Gushukang granules (Yi Yanghuo, cooked Rehmannia, bone Chiu Bu, Huang Mei, Salvia miltiorrhiza, fungus, cucumber seeds, etc.) (Liaoning Kangchen Pharmaceutical Co., LTD., Z190101084).

Reagents: BioTeke TRI pure (Beijing, lot No. RP1001); BioTeke Super M-MLV reverse transcriptase (Beijing, Lot No. PR6502); BioTeke RNase inhibitor (Beijing, batch No. RP5602); BioTeke 2 \times Power Taq PCR MasterMix (Beijing, Lot No. PR1702); BioTeke SYBR Green (Batch No. SY1020, Beijing); Primer numbers 114165#, 114165#, 114165#; Name Era F, Era R, Er β F, Er β R, β -actin F, β -actin R.

Sequence: TGTTTGCTCCTAACTTGCTCTT, CATGCGGAATCGACTTGAC
GCACCTGTCTCCTTTAGCG, GGCACAACCTGCTCCCACTA
GGAGATTACTGCCCTGGCTCCTAGC, GGCCGGACTCATCGTACTCCTGCTT,
primers length: 22,19,19,19,25. 25. Tm: 56.7, 56.7, 55.7, 56.4, 60.1, 62.

Product length: 104,151,155.

2.2. Experimental methods

Experimental animals: A total of 60 SD female rats, weighing 180 g–200 g, were provided by the Laboratory Animal Center of Jilin University with qualification certificate number: SCXK (Liao) 2015-0001. All SD rats were fed in a sterile environment with constant temperature and humidity, the room temperature was controlled at 23 °C–25 °C, the humidity was 55%–60%, the standard rat diet was fed, the food and water were freely consumed, and the ventilation was good. After 14 days of adaptive feeding, a retinoic acid-induced osteoporosis model was established. After 1 week of adaptive feeding, female SD rats were randomly divided into 6 groups: Blank control group, model group, positive drug group, and Chagan Gaoyou-4 powder low-dose, medium-dose, and high-dose groups, with 10 rats in each group. Except for the control blank group, the rats in the other groups were intragastric with 80 mg/kg retinoic acid every day [13], and the administration cycle was 14 days. After administration, rats in the blank group and model group were selected, BMD was measured by Dual-emission X-ray Absorptiometry (DXA) bone densitometer, serum alkaline phosphatase (ALP) level was measured, and external performance was evaluated whether the modeling was successful. After the success of the model, the positive drug group was given Gushukang granule suspension (2 g/kg) (Gushukang granule is a common traditional Chinese patent medicines and simple preparations in

the treatment of osteoporosis in traditional Chinese medicine. It can regulate the level of calcium and phosphorus in the body, enhance the activity of osteoblasts, promote bone repair, inhibit bone absorption, and improve bone density [17–19]. Because the effect of this formula on osteoporosis is similar to that of Chagan Gaoyou-4 in this study, and both formulas belong to the traditional ethnic medicine category, this medicine was selected as the positive drug group), the low, medium and high dose groups were given Chagan Gaoyou-4 powder (0.514 g/kg, 1.056 g/kg, 2.112 g/kg), respectively, the blank control group and the model group were given distilled water, and their body weight was weighed once a week. Adjust the dose according to the change in body mass. The rats were given continuous administration for 21 days. After the last administration for 2 h, blood was taken from the abdominal aorta after anesthesia, and serum was collected and immediately stored in an ultra-low temperature refrigerator at -80°C for later use. The right femur and spine were quickly removed, and bone mineral density was measured. The left femur was taken, and the upper soft tissue was cleaned, washed with 0.9% normal saline, and wrapped in sterile gauze for storage in liquid nitrogen. The expression of ER α and ER β protein in the femur tissues of each group was to be measured.

2.3. Main observation indicators

The changes in body hair color, consciousness, limb movement, food intake, and body mass were carefully observed before and after modeling and drug treatment.

2.3.1. Bone density test

After routine anesthesia, rats were sprawled on the scanning bed of the X-ray bone densitometer, and the software system of small animal bone density detection in the computer terminal was opened to conduct spine bone density analysis with the software of the instrument.

2.3.2. Determination of serum indexes

Blood samples were collected from the abdominal aorta, serum was separated, and Estradiol (E2) content in serum was detected by an automatic biochemical analyzer.

2.3.3. Viscera wet weight measurement

The uterus, adrenal glands, and spleen were taken and weighed after sacrificed the animal. The left femur was selected for RT-PCR detection and was quickly cleaned and stored in liquid nitrogen for future detection.

2.4. Statistical analysis

SPSS 23.0 software was used for data analysis, the data were expressed as $x \pm s$, and the comparison between groups was analyzed by ANOVA. $P < 0.05$ was considered a significant difference.

3. Results

3.1. Effects of Chagan Gaoyou-4 powder on spinal bone mineral density (BMD) in osteoporosis rats

The spinal BMD was measured as shown in **Table 1**. Compared with the normal group, BMD in the model group decreased after administration, and the difference was statistically significant ($P < 0.001$). After 21 days of administration, the BMD of the spine in Chagan Gaoyou-4 group was higher than the retinoic acid group ($P < 0.01$), and the BMD of the spine was also higher in Chagan Gaoyou-4 group ($P < 0.05$). The spinal BMD of Chagan Gaoyou-4 high-dose group and Gushukang granule group was slightly higher than the normal group, but there was no statistical significance ($P > 0.05$), and the BMD of the middle and low dose group was higher than the model group ($P < 0.05$).

Table 1. Effect of Chagan Gaoyou-4 powder on spinal bone mineral density in osteoporosis rats ($x \pm s$).

Group	Quantity	mg/cm ²
Normal	10	0.135 ± 0.003
Model	10	0.126 ± 0.002*
Positive	10	0.141 ± 0.002
Low dose	10	0.134 ± 0.002#
Medium dose	10	0.136 ± 0.002#
High dose	10	0.138 ± 0.003#

* $P < 0.05$ vs. Normal group, # $P < 0.05$ vs. Model group.

3.2. Effects of Chagan Gaoyou-4 powder on wet weight of liver, spleen, kidney, adrenal gland, and uterus in rats

The wet weight measurements of the liver, spleen, kidney, adrenal gland, and uterus of rats are shown in **Table 2**. Compared with the normal group, both the uterus and adrenal gland of the model group were decreased after administration, and the difference was statistically significant ($P < 0.05$, $P < 0.001$), indicating that the rat model induced by retinoic acid had a damaging effect on the uterus and adrenal gland, while the wet weight of spleen was decreased. But there was no statistical significance ($P > 0.05$).

Table 2. Effects of Chagan Gaoyou-4 powder wet-weight powder on the ovary, kidney, adrenal gland, spleen, and liver of rats ($x \pm s$).

Group	Liver (mg)	Spleen (mg)	Kidneys (mg)	Adrenal glands (mg)	Uterus (mg)
Normal	8.7447 ± 0.70	0.5939 ± 0.04	1.7454 ± 0.03	0.1024 ± 0.005	0.2201 ± 0.005
Model	7.2648 ± 0.34*	0.5000 ± 0.03*	1.6960 ± 0.02*	0.0834 ± 0.002*	0.1697 ± 0.004*
Positive	7.8978 ± 0.38	0.5822 ± 0.01	1.6990 ± 0.04	373.97 ± 13.08	0.1871 ± 0.004
Low dose	7.5565 ± 0.37#	0.5138 ± 0.03#	1.6836 ± 0.03#	0.0853 ± 0.005#	0.1692 ± 0.004#
Medium dose	7.9358 ± 0.62#	0.5342 ± 0.01#	1.6925 ± 0.02#	0.0861 ± 0.004#	0.1877 ± 0.004#
High dose	8.1771 ± 0.53#	0.5787 ± 0.03#	1.7214 ± 0.03#	0.0986 ± 0.005#	0.2134 ± 0.004#

* $P < 0.05$ vs. Normal group, # $P < 0.05$ vs. Model group.

3.3. Effects of Chagan Gaoyou-4 powder on serum E2 level and receptor expression in osteoporosis rats

The serum E2 content and the expression of ER α and ER β protein in bone tissue

were measured as shown in **Table 3**. Compared with the corresponding normal group, the serum E2 content in the model group was significantly decreased after administration ($P < 0.01$). Compared with the model group, E2 content in all treatment groups was increased ($P < 0.05$), which was higher in the normal group, control group, and Chagan Gaoyou-4 group.

E2 in the normal group was higher than that in the two groups, but the difference was not statistically significant ($P > 0.05$). As shown in **Table 3**, compared with the normal group after administration, the relative expression levels of ER α and ER β proteins in the model group were decreased, with statistical significance ($P < 0.001$), and the relative expression levels of proteins in each administration group were significantly increased compared with the model group, with statistical significance ($P < 0.001$).

After 21 days of administration, there was no significant difference in the levels of two proteins between the normal group the control group, and the dose group ($P > 0.05$). The ER α of Chagan Gaoyou-4 is expressed as ER β . The expression of Chagan Gaoyou-4 increased with increasing dose at medium and high doses, but no statistical significance at low doses.

Table 3. Effects of Chagan Gaoyou-4 powder on serum estrogen level and receptor expression in osteoporosis rats ($x \pm s$).

Group	E2 (ng/L)	ER α	ER β
Normal	224.71 \pm 4.94	1.00 \pm 0.05	1.00 \pm 0.03
Model	149.99 \pm 5.50*	0.21 \pm 0.02*	0.25 \pm 0.02*
Positive	169.72 \pm 2.88	0.71 \pm 0.05	0.86 \pm 0.07
Low dose	187.59 \pm 5.51#	0.25 \pm 0.03#	0.22 \pm 0.02#
Medium dose	203.05 \pm 2.89#	0.38 \pm 0.01#	0.34 \pm 0.01#
High dose	214.05 \pm 4.59#	0.60 \pm 0.04#	0.45 \pm 0.02#

* $P < 0.05$ vs. Normal group, # $P < 0.05$ vs. Model group.

4. Discussion

Traditional medicine believes that osteoporosis is based on the deficiency of kidney essence, bone depletion, and marrow reduction, and the main pathogenesis of blood obstruction and bone dysfunction [20]. Under the guidance of the above theories and combined with practical clinical application, Chagan-Gaoyou-4 powder has a good clinical effect. Chagan Gaoyou-4 powder, Mongolian medicine compound preparation is developed by the Mongolian medicine preparation Room of the Affiliated Hospital of Inner Mongolia University for Nationalities. Some studies have shown that compound preparation has the function of tonifying the kidney, spleen, calcium, and bone marrow.

The results of this experiment showed that Chagan Gaoyou-4 could effectively increase serum E2 level in rats induced by retinoic acid ($P < 0.01$, $P < 0.05$), increase the relative expression of ER α and ER β protein, and increase BMD, which was consistent with the results of previous studies on the treatment of osteoporosis with Mongolian medicine [21]. In addition, Chagan Gaoyou-4 could increase spinal BMD, significantly increase the expression levels of ER α and ER β protein ($P < 0.001$), and

increase the level of serum E2 ($P < 0.05$). Retinoic acid can effectively induce the improvement of bone mineral density in rats, which is related to the estrogen-like supplementation of Chagan Gaoyou-4 after the decrease of E2 induced by retinoic acid in rats with osteoporosis. E2 in serum can promote the formation of osteoblasts, inhibit the formation of osteoclasts, and improve the function of bone mineralization [22–26]. The results of serum E2 showed that the serum E2 level of Chagan Gaoyou-4 group increased after administration ($P < 0.01$, $P < 0.05$), and the difference was not significant compared with the other two administration groups, indicating that Chagan Gaoyou-4 may have an estrogen-like effect, and the improvement of estrogen level in osteoporosis rats is similar to Gushukang granules. Gushukang granule is a traditional drug commonly used in clinical prevention and treatment of osteoporosis [27,28]. Experimental results showed that continuous administration of Chagan Gaoyou-4 led to an increase in BMD in the spine of rats ($P < 0.01$). The BMD of the neck of femur could also be improved ($P < 0.05$). There was no statistical significance in BMD of Chagan Gaoyou-4 group compared with normal group and control group ($P > 0.05$). At the same time, the levels of ER α and ER β protein in Chagan Gaoyou-4 group were significantly higher than those in model group ($P < 0.001$). Compared with the administration group, the protein levels of ER α and ER β were lower in the model group ($P < 0.05$).

It can be seen that Mongolian medicine Chagangaoyou-4 powder can effectively increase the level of E2 in serum, affect the expression of ER α and ER β protein in bone tissue after a period of application [18,27], improve bone mineral density, and thus restore the bone metabolism level induced by retinoic acid in rats to a certain extent by stimulating estrogen receptor signaling pathway [29–31]. The role of treating osteoporosis. In terms of the increase in visceral wet weight, it may have a certain protective effect on the reproductive system and digestive systems, but this needs further research.

From the comprehensive analysis of all aspects, it can be seen that the change of estrogen level in rats affects the expression degree of ER α and ER β to some extent, and the BMD value also changes. The comparison between the plant Mongolian medicine Chagan Gaoyou-4 group and Gushukang granule group showed that both Gushukang granule and Mongolian medicine Chagan Gaoyou-4 powder could up-regulate the imbalance of bone metabolism induced by accutane in rats to a certain extent, which was consistent with the mechanism used in clinical prevention and treatment of osteoporosis. Mongolian medicine Chagan Gaoyou-4 powder is a compound preparation of Mongolian medicine extracted from the treasure house of traditional medicine in China after long-term practice. It has a certain estrogen-like effect and contains calcium-containing single drug cold water stone. After being processed in combination with traditional Mongolian medicine, it is good for nourishing bones. It has great potential in preventing and treating OP. However, there are still many shortcomings in this study, such as an insufficient number of experimental samples, a short experimental period, and little morphological evidence, which may have a certain impact on the experimental results. Our team will increase the sample size and supplement morphological experiments in the next step of research, including HE staining, immunohistochemical observation of rat ER α , ER β protein expression, to provide a research basis for the clinical promotion and application of

Chagan Gaoyou-4 powder.

Author contributions: Methodology, SS; software, DS; resources, T; data curation, DS; writing—original draft preparation, SS; writing—review and editing, DS. All authors have read and agreed to the published version of the manuscript.

Ethical approval: The study was conducted in accordance with the Declaration of Helsinki, and approved by the Ethics Committee of Affiliated hospital of Inner Mongolia University for The Nationalities, NM-LL-2018-10-20-01 (20 October 2018).

Conflict of interest: The authors declare no conflict of interest.

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